NADPH regulates human NAD kinase, a NADP-biosynthetic enzyme.

AUTHOR(S):

Ohashi, Kazuto; Kawai, Shigeyuki; Koshimizu, Mari; Murata, Kousaku

CITATION:

Ohashi, Kazuto ...[et al]. NADPH regulates human NAD kinase, a NADP-biosynthetic enzyme.. Molecular and cellular biochemistry 2011, 355(1-2): 57-64

ISSUE DATE:

2011-09

URL:

http://hdl.handle.net/2433/147259

Title

NADPH regulates human NAD kinase, a $NADP^+$-biosynthetic enzyme

Author names and affiliations

Kazuto Ohashi, Shigeyuki Kawai, Mari Koshimizu, Kousaku Murata*

Laboratory of Basic and Applied Molecular Biotechnology, Division of Food and Biological Science, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan

***Corresponding author**

Kousaku Murata

Address: Laboratory of Basic and Applied Molecular Biotechnology, Division of Food and Biological Science, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan

Telephone number: +81-774-38-3766

Fax number: +81-774-38-3767

E-mail: kmurata@kais.kyoto-u.ac.jp

Abstract

NAD kinase (NADK, EC 2.7.1.23) is the sole $NADP^+$-biosynthetic enzyme that catalyzes phosphorylation of NAD^+ to yield $NADP^+$ using ATP as a phosphoryl donor, and thus, plays a vital role in the cell and represents a potentially powerful antimicrobial drug target. Although methods for expression and purification of human NADK have been previously established (F. Lerner, M. Niere, A. Ludwig, M. Ziegler, Biochem. Biophys. Res. Commun. 288 (2001) 69-74), the purification procedure could be significantly improved. In this study, we improved the method for expression and purification of human NADK in *Escherichia coli* and obtained a purified homogeneous enzyme only through heat treatment and single column chromatography. Using the purified human NADK, we revealed a sigmoidal kinetic behavior toward ATP and the inhibitory effects of NADPH and NADH, but not of $NADP^+$, on the catalytic activity of the enzyme. These inhibitory effects provide insight into the regulation of intracellular NADPH synthesis. Furthermore, these attributes may provide a clue to design a novel drug against *Mycobacterium tuberculosis* in which this bacterial NADK is potently inhibited by $NADP^+$.

Keywords

NAD kinase; human; drug design; $NADP^+$; NADPH; NADH

Abbreviations

NADK, NAD kinase; LB, Luria-Bertani; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Introduction

NAD^+ and $NADP^+$ are cofactors involved in oxidative–reductive reactions [1, 2]. The function of NAD(H) (NAD^+ and NADH) is distinguishable from that of NADP(H) ($NADP^+$ and NADPH). NAD(H) mainly functions in catabolic reactions, while NADP(H) is involved in anabolic reactions and defense against oxidative stress [3]. NAD^+ also functions as a substrate for mono- and poly-ADP-ribosylation and is involved in cyclic ADP-ribose synthesis [2] and histone deacetylation [4]. $NADP^+$ functions as a substrate in the synthesis of nicotinic acid adenine dinucleotide phosphate, which is a potent intracellular Ca^{2+}-mobilizing messenger [1, 2, 5].

NAD kinase (NADK, EC 2.7.1.23) is the sole $NADP^+$-biosynthetic enzyme that catalyzes phosphorylation of NAD^+ to yield $NADP^+$ using ATP as a phosphoryl donor [1], and thus, plays a vital role in the cell. Accordingly, NADK has been found to be essential for many microbes, including *Mycobacterium tuberculosis* [6], *Bacillus subtilis* [7], *Staphylococcus aureus* [8], *Haemophilus influenzae* [8], *Escherichia coli* [8, 9], *Streptococcus pneumoniae* [8], *Salmonella enterica* [9, 10], and *Saccharomyces cerevisiae* [11, 12], thus representing a potentially powerful antimicrobial drug target [13, 14]. Several bacterial recombinant NADKs have been characterized in detail, including those from *E. coli* [15], *B. subtilis* [16], and *M. tuberculosis* [17, 18]. Moreover, tertiary structures of NADKs from the pathogenic bacteria *M. tuberculosis* [19, 20] and *Listeria monocytogenes* [21] have been published. However, the tertiary structure of human NADK has not yet been reported.

Lerner *et al.* [22] established methods for expression and purification of human NADK and determined some properties of this enzyme [22]. Although they provided a detailed purification procedure, the procedure still has needed several steps such as heat treatment followed by DEAE column chromatography and nickel–nitrilotriacetic acid column chromatography. Thus, an improvement in the methods for expression and purification of human NADK would facilitate further study of this vital enzyme. Further determination of the structure and catalytic properties of human NADK would provide a good foundation for designing a novel antimicrobial drug [13].

In this article, we describe improved methods to express and purify human NADK, thus enabling to obtain larger amounts of the purified homogeneous enzyme. In addition, we reveal some kinetic properties of human NADK that will provide insight into the regulation of intracellular NADPH synthesis and possibly a clue to design a novel antimicrobial drug.

Materials and methods

Strains and plasmids

As a host for plasmid amplification, *E. coli* DH5α and JM109 were routinely cultured at 37 °C in Luria-Bertani (LB) medium containing 1% tryptone, 0.5% yeast extract, 1% NaCl, and ampicillin (100 μg/ml) (pH 7.2). *E. coli* M15 (pREP4) and SG13009 (pREP4) were purchased from Qiagen (Hilden, Germany), and *E. coli* Rosetta-gami, Rosetta-gami(DE3), Rosetta-gami(DE3)pLysS, Rosetta-gami 2(DE3),

and Rosetta-gami B(DE3) were obtained from Novagen (Darmstadt, Germany). The culture conditions for these *E. coli* strains are described below.

Human NADK cDNA was amplified by PCR using human leukocyte cDNA (QUICK-Clone cDNA, Clontech, shiga, Japan) as a template and primers [HsNADKBamHI-f, CGGGATCCATGGAAATGGAACAAGAAAAAATGAC (the *Bam*HI site is underlined) and HsNADK-C(NoRe)-r, CTAGCCCTCCTCCTCCTCCTC], and was inserted into the *Hinc*II site of pUC18, resulting in pMK1966. Human NADK cDNA obtained by digestion of pMK1966 with *Bam*HI was inserted into the *Bam*HI site of pQE-30 (Qiagen), yielding pMK2071. The primary structure encoded by the cloned cDNA was identical to that of the cDNA sequence (with Phe $(Glu)_9$ Gly as C-terminus) deposited in the NCBI database (accession no. NP_001185922), except that the former contains one additional Glu residue at its C-terminus, i.e., it has Phe $(Glu)_{10}$ Gly.

A DNA fragment encoding for an N-terminal sequence consisting of 87 amino acid residues of human NADK was excised from the full-length cDNA clone pMK2071, resulting in pMK2784 that encodes for deleted human NADK with an N-terminal sequence, ^{88}MHIQDPASQRL^{98}. This deletion was generated by inverse PCR using primers (5′-CACATTCAGGACCCCGCGAG-3′ and 5′-CATATGTCCGTGATGGTGATGGTG-3′). The recombinant proteins encoded by pMK2071 and pMK2784 contained a His-tag (MRGSHHHHHHGS) at their N-terminal sites.

To express human NADK cDNA, pMK2071 was introduced into *E. coli* M15 (pREP4), SG13009 (pREP4), and Rosetta-gami, yielding MK2105, MK2106, and MK2107, respectively. pMK2784 was introduced into *E. coli* Rosetta-gami(DE3),

Rosetta-gami(DE3)pLysS, Rosetta-gami 2(DE3), and Rosetta-gami B(DE3), yielding MK2801, MK2802, MK2803, and MK2804, respectively.

Expressions

The cells were precultured overnight in LB media supplemented with 100 μg/ml ampicillin and 25 μg/ml kanamycin (MK2105 and MK2106) or with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol (MK2107, MK2801, MK2802, MK2803, and MK2804). The cells were collected, inoculated in LB media supplemented with the same antibiotics, and aerobically cultured at 37 °C until A_{600} reached 0.50–1.0. Isopropyl-β-D-thiogalactopyranoside was then added to obtain a final concentration of 0.025 or 1.0 mM in order to induce expression of human NADK. Cell cultivation was continued further at 16 or 37 °C under aerobic conditions for 4, 8, 12, or 24 h. To purify human NADK, MK2107 and MK2802 were cultivated at 37 °C for 8 h (MK2107) or 12 h (MK2802) after the addition of isopropyl-β-D-thiogalactopyranoside at 1.0 mM. To prepare the crude extract, cells were collected by centrifugation, suspended in 10 mM Tris-HCl (pH8.0), and sonicated to yield cell lysate. Before and after sonication, phenylmethylsulfonyl fluoride was added to obtain a final concentration of 1.0 mM. After centrifugation of the lysate at 20,000 ×g and 4 °C for 10 min, the supernatant was used as crude extract.

Assays

NADK activity of human NADK was assayed at 37 °C by the modified continuous method [23]. Briefly, NADPH formation was continuously measured at A_{340} in a reaction mixture (1.0 ml) containing 5.0 mM NAD^+, 5.0 mM ATP, 5.0 mM glucose-6-phosphate, 0.5 U glucose-6-phosphate dehydrogenase (Sigma, St. Louis, MD, USA), 5.0 mM $MgCl_2$, and 100 mM Tris-HCl (pH 8.0). In the purified enzyme assay, $MgCl_2$ was used at 10 mM. Enzyme activity was assayed by a stop method [23] to determine inhibitory effects and optimum pH and temperature conditions. Briefly, the amount of $NADP^+$ formed was enzymatically determined using 0.5 U glucose-6-phosphate dehydrogenase after the reaction was terminated by immersing the test tube in boiling water for 5 min. Glucose-6-phosphate and glucose-6-phosphate dehydrogenase were removed from the reaction mixture as described above. When the inhibitory effects of NADP(H) and NADH were investigated, control reactions without human NADK were simultaneously performed in every assay.

NADH kinase activity was assayed in a reaction mixture (1.0 ml) containing 2.0 mM NADH, 5.0 mM ATP, 10.0 mM $MgCl_2$, and 100 mM Tris-HCl (pH 8.0) [23]. One unit of enzyme activity was defined as 1.0 µmol NADP(H) produced in 1 min at 37 °C, and specific activity was expressed in U/mg protein. Protein concentrations of the crude extract and purified human NADK from MK2107 were determined by the Bradford method [24]. Protein concentrations of purified human NADK from MK2802 were determined using the extinction coefficient (33,920) at A_{280}. The extinction coefficients were calculated using the ProtParam tool on the ExPASy

server (http://ca.expasy.org/tools/protparam.html).

Purification

Centrifugation was performed at 20,000 ×*g* and 4 °C for 10 min. Human NADK was expressed from MK2107 cells cultured in 2 l LB (0.5 l per 2 l Erlenmeyer flask) as described above and treated as described [22]. This was followed by Mono Q chromatography. The partially purified human NADK was applied to Mono Q 5/50 GL (0.5 × 5.0 cm) (GE Healthcare, Buckinghamshire, England) equilibrated with buffer A (20 mM Tris-HCl (pH 8.5), 0.10 mM NAD^+, 0.50 mM dithiothreitol, 1.0 mM ethylenediaminetetraacetic acid, and 0.10 mM pepstatin A). After washing the column with 18 ml buffer A, elution was performed with a linear gradient of NaCl from 0 to 1.0 M in buffer A. Fructions that were obtained by an elution at 370–470 mM NaCl were combined, dialyzed against buffer A, and used as purified human NADK.

Human NADK was purified from MK2802 as stated below. Crude extract was prepared from MK2802 cells cultured in 13.5 l LB (1.5 l per 2 l Sakaguchi flask) and was treated as described above. Samples were heated at 60 °C for 5 min and centrifuged. The supernatant was applied to a TALON column (1.5 × 22 cm) (Clontech) equilibrated with 10 mM Tris-HCl (pH8.0). The column was washed with 2 l of 10 mM Tris-HCl (pH8.0) containing 0.30 M NaCl. Human NADK was eluted with 10 mM Tris-HCl (pH8.0) containing 0.30 M NaCl and 150 mM imidazole. Fractions were combined, dialyzed against 10 mM Tris-HCl (pH 8.0), and used as purified human NADK. Alternatively, the crude extract prepared from MK2802 cells

was treated as mentioned above and was directly applied to the TALON column without heat treatment. The column was washed with 2 l of 10 mM Tris-HCl (pH8.0) containing 0.30 M NaCl and 30 mM imidazole, and eluted as above.

Other analytical methods

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 12.5% polyacrylamide gel [25]. Proteins in the gel were visualized with Coomassie brilliant blue R-250. The molecular mass of the enzyme was estimated by gel filtration chromatography as described [26]; however, 10 mM Tris-HCl (pH 8.0) containing 0.15 M NaCl was used as an elution buffer. For the N-terminal amino acid sequence, purified and unpurified enzymes were transferred to a polyvinylidene difluoride membrane and analyzed using a Procise 492 protein sequencing system (Applied Biosystems Division of Perkin-Elmer, Foster City, CA, USA).

Results and discussion

Improved methods to express and purify human NADK

According to the method described by Lerner *et al.* [22], we expressed the full-length human NADK cDNA cloned into pQE-30 (pMK2071) using an *E. coli* M15 (pREP4) host cell and obtained 0.23 U/mg NADK activity in the crude extract (Table 1). To improve expression, effects of the host cells (*E. coli* SG13009 (pREP4)

or Rosetta-gami), concentrations of isopropyl-β-D-thiogalactopyranoside (0.025 or 1.0 mM), and induction conditions (16 or 37 °C and 4, 8, 12, or 24 h) were examined. Maximum expression (1.02 U/mg) was obtained in the crude extract when expression was induced at 37 °C for 8 h with 1.0 mM isopropyl-β-D-thiogalactopyranoside using *E. coli* Rosetta-gami (Table 1). The expressed human NADK was treated according to the purification procedure described by Lerner *et al*. [22]. However, this procedure yielded an unpurified enzyme. The unpurified enzyme was further purified by Mono Q chromatography as described in Materials and methods. The purified enzyme (0.39 mg; 7.21 U/mg) was obtained from a 2 l culture. SDS-PAGE analysis showed that the subunit molecular mass of the purified enzyme was 43 kDa, which was smaller than that predicted (49 kDa) from the primary structure (data not shown). Analysis of the N-terminal amino acid sequence of the purified enzyme confirmed that the enzyme lacked the N-terminal sequences consisting of 60, 63, and 74 amino acid residues of human NADK, indicating the heterogeneity of the purified enzyme.

To circumvent this problem, the DNA region that encoded the N-terminal sequence consisting of 87 amino acid residues of human NADK was excised from the full-length human NADK cDNA as described in Materials and methods. The deleted human NADK was expressed in *E. coli* Rosetta-gami(DE3), and it showed a specific activity of 0.79 U/mg (Table 1). Further optimization of the expression condition revealed that induction at 37 °C for 8–12 h in the presence of 1.0 mM isopropyl-β-D-thiogalactopyranoside using *E. coli* Rosetta-gami(DE3)pLysS as a host cell gave the maximum specific activity (4.29 U/mg). This activity was improved by approximately 18.7-fold compared with our initial attempt (0.23 U/mg;

Table 1). Moreover, we found that the heat treatment, followed by TALON column chromatography was sufficient to obtain the purified enzyme (9.34 U/mg, Fig. 1a). The subunit molecular mass of the purified enzyme (43 kDa) was in good agreement with the predicted mass (41 kDa). The N-terminal amino acid sequence of the enzyme was RGSHH, which corresponded to that of the His-tag, confirming the homogeneity of the purified enzyme. In all purification experiments, the purified enzyme (15 mg) was routinely obtained from a 13.5 l culture. Thus, we improved the methods for expression and purification of human NADK (Table 2, Fig. 1a).

We also found that the enzyme could be purified only through TALON column chromatography without heat treatment (Fig. 1b). However, the purified NADK without heat treatment yielded three peaks (representing 210, 400, and 650 kDa) on gel filtration chromatography; each peak showed NADK activity. NADK in each peak was not divided into three peaks on repeated gel filtration chromatography (data not shown), thus indicating the heterogeneity in the quaternary structure of this human NADK. In contrast, the purified enzyme obtained by heat treatment, which was followed by TALON column chromatography (Fig. 1a), yielded a single peak representing 172 kDa (data not shown). Thus, the procedure using heat treatment was considered preferable for enzyme purification, and the properties of human NADK purified by this procedure were determined.

Properties of human NADK

Although the properties of purified human NADK were similar to those reported [22] (Table 3), we found that human NADK exhibits sigmoidal kinetic behavior

toward ATP, while saturation curve toward NAD^+ (Fig. 2). The sigmoidal kinetic behavior was emphasized by a Hanes–Woolf plot (Fig. 2b). The sigmoidal kinetic behavior toward ATP was also observed in NADKs from *B. subtilis* [16] and *M. tuberculosis* [18]. With regard to NAD^+, *M. tuberculosis* NADK shows sigmoidal kinetic behavior [18], whereas *B. subtilis* NADK exhibits a saturation curve [16].

NADH-phosphorylating (NADH kinase) activity of recombinant human NADK was qualitatively, but not quantitatively, demonstrated by Pollak *et al.* [27]. We showed that human NADK exhibited NADH kinase activity (0.6 U/mg), accounting for only 5.0% of NADK activity (11.9 U/mg) determined in the presence of 2.0 mM NAD^+. Some bacterial NADKs, such as those from *M. tuberculosis*, utilize inorganic polyphosphate as a phosphoryl donor [17, 18]. Inorganic polyphosphate is a polymer of inorganic orthophosphate residues linked by a high-energy phosphoanhydride bond [28], and is available as metaphosphate, hexametaphosphate, and tetrapolyphosphate. The relative activities of purified human NADK in the presence of 1 mg/ml metaphosphate, 1 mg/ml hexametaphosphate, and 5 mM tetrapolyphosphate were 2.3, 1.9, and 0.5%, respectively, compared with NADK activity assayed in the presence of 5.0 mM ATP.

Inhibitory effects of NADPH and NADH, but not of $NADP^+$

Determining how human NADK is regulated by $NADP^+$, NADPH, and NADH is a crucial step in understanding how human NADK influences the intracellular redox balance. Although $NADP^+$ exhibited no inhibitory effect on NADK activity of human NADK, NADPH and NADH showed competitive inhibitions with K_i values

of 0.13 mM for NADPH and 0.34 mM for NADH (Table 4, Fig. 3, Online Resource Fig. S1). This agrees with a previous report, which shows that NADK activity of partially purified pigeon liver NADK is competitively inhibited by NADPH and NADH, while the effect of $NADP^+$ on NADK activity of the enzyme was not investigated [29]. Although the information regarding the effect of these nucleotides on NADK activity has been limited, *M. tuberculosis* NADK has been reported to be inhibited by NADH, $NADP^+$, and NADPH, wherein $NADP^+$ is the most potent inhibitor [18]. The residual NADK activity of *M. tuberculosis* NADK was 22, 43, and 50% in the presence of 0.40 mM $NADP^+$, NADPH, and NADH, respectively, although the inhibitory mechanism has not been investigated [18]. The fact that $NADP^+$ inhibits *M. tuberculosis* NADK but not human NADK is a clue to design a novel antimicrobial drug, i.e., an analog of $NADP^+$, which would possibly inhibit only *M. tuberculosis* NADK, thereby being lethal to the bacterium and not the host.

Recently, Pollak *et al.* [27] established human cell lines overexpressing human NADK (NADK(+) cells). In NADK(+) cells that showed approximately 180-fold higher NADK activity than control cells, the NADPH level increased by 4- to 5-fold, and not 180-fold, while the $NADP^+$ level was not prominently altered. The authors suggested that human NADK may be inhibited by NADPH. Our data demonstrating the inhibition of human NADK by NADPH convincingly support their suggestion. Taken together the low NADH kinase activity of human NADK, NADPH would be produced by $NADP^+$-dependent dehydrogenases through reduction of $NADP^+$. The fact that human NADK is localized in the cytosol [27] and that NADPH, but not $NADP^+$, is significant for the regulation of human NADK would indicate that the function of cytosolic $NADP^+$-dependent dehydrogenases is important for the

regulation of $NADP^+$ synthesis (NADK activity), although the physiological role of inhibition of human NADK by NADH remains unclear.

NADPH is required to protect cells against oxidative stress, and cytosolic $NADP^+$-dependent glucose-6-phosphate dehydrogenase in human cells is induced when exposed to oxidative stress [30]. When cells survive against oxidative stress, intracellular NADPH is consumed and resynthesized quickly, while human NADK synthesizes $NADP^+$ to guarantee the intracellular requirement for NADPH. When oxidative stress is removed, the abundant NADPH inhibits $NADP^+$ synthesis to prevent accumulation of excess $NADP^+$ and NADPH, thus resulting in the maintenance of homeostasis of intracellular $NADP^+$ and NADPH.

Acknowledgments

This work was supported in part by a Grant-in-Aid for SK from the Ministry of Education, Culture, Sports, Science and Technology of Japan (21780069).

References

1. Pollak N, Dolle C, Ziegler M (2007) The power to reduce: pyridine nucleotides - small molecules with a multitude of functions. Biochem J 402 (2):205-218
2. Ziegler M (2000) New functions of a long-known molecule. Emerging roles of NAD in cellular signaling. Eur J Biochem 267 (6):1550-1564
3. Outten CE, Culotta VC (2003) A novel NADH kinase is the mitochondrial source of NADPH in *Saccharomyces cerevisiae*. EMBO J 22 (9):2015-2024
4. Smith JS, Brachmann CB, Celic I, Kenna MA, Muhammad S, Starai VJ, Avalos

JL, Escalante-Semerena JC, Grubmeyer C, Wolberger C, Boeke JD (2000) A phylogenetically conserved NAD^+-dependent protein deacetylase activity in the Sir2 protein family. Proc Natl Acad Sci USA 97 (12):6658-6663

5. Yamasaki M, Masgrau R, Morgan AJ, Churchill GC, Patel S, Ashcroft SJ, Galione A (2004) Organelle selection determines agonist-specific Ca^{2+} signals in pancreatic acinar and beta cells. J Biol Chem 279 (8):7234-7240

6. Sassetti CM, Boyd DH, Rubin EJ (2003) Genes required for mycobacterial growth defined by high density mutagenesis. Mol Microbiol 48 (1):77-84

7. Kobayashi K, Ehrlich SD, Albertini A, Amati G, Andersen KK, Arnaud M, Asai K, Ashikaga S, Aymerich S, Bessieres P, Boland F, Brignell SC, Bron S, Bunai K, Chapuis J, Christiansen LC, Danchin A, Debarbouille M, Dervyn E, Deuerling E, Devine K, Devine SK, Dreesen O, Errington J, Fillinger S, Foster SJ, Fujita Y, Galizzi A, Gardan R, Eschevins C, Fukushima T, Haga K, Harwood CR, Hecker M, Hosoya D, Hullo MF, Kakeshita H, Karamata D, Kasahara Y, Kawamura F, Koga K, Koski P, Kuwana R, Imamura D, Ishimaru M, Ishikawa S, Ishio I, Le Coq D, Masson A, Mauel C, Meima R, Mellado RP, Moir A, Moriya S, Nagakawa E, Nanamiya H, Nakai S, Nygaard P, Ogura M, Ohanan T, O'Reilly M, O'Rourke M, Pragai Z, Pooley HM, Rapoport G, Rawlins JP, Rivas LA, Rivolta C, Sadaie A, Sadaie Y, Sarvas M, Sato T, Saxild HH, Scanlan E, Schumann W, Seegers JF, Sekiguchi J, Sekowska A, Seror SJ, Simon M, Stragier P, Studer R, Takamatsu H, Tanaka T, Takeuchi M, Thomaides HB, Vagner V, van Dijl JM, Watabe K, Wipat A, Yamamoto H, Yamamoto M, Yamamoto Y, Yamane K, Yata K, Yoshida K, Yoshikawa H, Zuber U, Ogasawara N (2003) Essential *Bacillus subtilis* genes. Proc Natl Acad Sci USA 100 (8):4678-4683

8. Zalacain M, Biswas S, Ingraham KA, Ambrad J, Bryant A, Chalker AF, Iordanescu S, Fan J, Fan F, Lunsford RD, O'Dwyer K, Palmer LM, So C, Sylvester D, Volker C, Warren P, McDevitt D, Brown JR, Holmes DJ, Burnham MK (2003) A global approach to identify novel broad-spectrum antibacterial targets among proteins of unknown function. J Mol Microbiol Biotechnol 6 (2):109-126

9. Gerdes SY, Scholle MD, D'Souza M, Bernal A, Baev MV, Farrell M, Kurnasov OV, Daugherty MD, Mseeh F, Polanuyer BM, Campbell JW, Anantha S, Shatalin KY, Chowdhury SA, Fonstein MY, Osterman AL (2002) From genetic footprinting to antimicrobial drug targets: examples in cofactor biosynthetic pathways. J Bacteriol 184 (16):4555-4572

10. Grose JH, Joss L, Velick SF, Roth JR (2006) Evidence that feedback inhibition of NAD kinase controls responses to oxidative stress. Proc Natl Acad Sci USA 103 (20):7601-7606

11. Miyagi H, Kawai S, Murata K (2009) Two sources of mitochondrial NADPH in the yeast *Saccharomyces cerevisiae*. J Biol Chem 284 (12):7553-7560

12. Bieganowski P, Seidle HF, Wojcik M, Brenner C (2006) Synthetic lethal and biochemical analyses of NAD and NADH kinases in *Saccharomyces cerevisiae* establish separation of cellular functions. J Biol Chem 281 (32):22439-22445

13. Magni G, Di Stefano M, Orsomando G, Raffaelli N, Ruggieri S (2009) NAD(P) biosynthesis enzymes as potential targets for selective drug design. Curr Med Chem 16 (11):1372-1390

14. Bi J, Wang H, Xie J (2011) Comparative genomics of NAD(P) biosynthesis and novel antibiotic drug targets. J Cell Physiol 226 (2):331-340.

doi:10.1002/jcp.22419

15. Kawai S, Mori S, Mukai T, Hashimoto W, Murata K (2001) Molecular characterization of *Escherichia coli* NAD kinase. Eur J Biochem 268 (15):4359-4365

16. Garavaglia S, Galizzi A, Rizzi M (2003) Allosteric regulation of *Bacillus subtilis* NAD kinase by quinolinic acid. J Bacteriol 185 (16):4844-4850

17. Kawai S, Mori S, Mukai T, Suzuki S, Yamada T, Hashimoto W, Murata K (2000) Inorganic polyphosphate/ATP-NAD kinase of *Micrococcus flavus* and *Mycobacterium tuberculosis* H37Rv. Biochem Biophys Res Commun 276 (1):57-63

18. Raffaelli N, Finaurini L, Mazzola F, Pucci L, Sorci L, Amici A, Magni G (2004) Characterization of *Mycobacterium tuberculosis* NAD kinase: functional analysis of the full-length enzyme by site-directed mutagenesis. Biochemistry 43 (23):7610-7617

19. Mori S, Yamasaki M, Maruyama Y, Momma K, Kawai S, Hashimoto W, Mikami B, Murata K (2005) NAD-binding mode and the significance of intersubunit contact revealed by the crystal structure of *Mycobacterium tuberculosis* NAD kinase-NAD complex. Biochem Biophys Res Commun 327 (2):500-508

20. Garavaglia S, Raffaelli N, Finaurini L, Magni G, Rizzi M (2004) A novel fold revealed by *Mycobacterium tuberculosis* NAD kinase, a key allosteric enzyme in NADP biosynthesis. J Biol Chem 279 (39):40980-40986

21. Poncet-Montange G, Assairi L, Arold S, Pochet S, Labesse G (2007) NAD kinases use substrate-assisted catalysis for specific recognition of NAD. J Biol Chem 282 (47):33925-33934

22. Lerner F, Niere M, Ludwig A, Ziegler M (2001) Structural and functional characterization of human NAD kinase. Biochem Biophys Res Commun 288 (1):69-74

23. Shi F, Kawai S, Mori S, Kono E, Murata K (2005) Identification of ATP-NADH kinase isozymes and their contribution to supply of NADP(H) in *Saccharomyces cerevisiae*. FEBS J 272 (13):3337-3349

24. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254

25. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227 (5259):680-685

26. Ochiai A, Mori S, Kawai S, Murata K (2004) Overexpression, purification, and characterization of ATP-NAD kinase of *Sphingomonas* sp. A1. Protein Expr Purif 36 (1):124-130

27. Pollak N, Niere M, Ziegler M (2007) NAD kinase levels control the NADPH concentration in human cells. J Biol Chem 282 (46):33562-33571

28. Kornberg A, Rao NN, Ault-Riche D (1999) Inorganic polyphosphate: a molecule of many functions. Annu Rev Biochem 68:89-125

29. Apps DK (1968) Kinetic studies of pigeon liver NAD kinase. Eur J Biochem 5 (3):444-450

30. Ursini MV, Parrella A, Rosa G, Salzano S, Martini G (1997) Enhanced expression of glucose-6-phosphate dehydrogenase in human cells sustaining oxidative stress. Biochem J 323 (Pt 3):801-806

Figure legends

Fig. 1. SDS-PAGE of purified human NADK. (a) Purified human NADK (110 μg protein). (b) Purified human NADK obtained without heat treatment (60 μg protein). Arrowheads indicate the positions of the purified human NADK.

Fig. 2. Kinetic analysis of human NADK. (a) The effect of substrate concentration ([ATP] or [NAD^+], mM) on the initial velocity (V, U/mg) of the reaction was assayed in the presence of 5 mM NAD^+ and various concentrations of ATP (closed squares) or 5 mM ATP and various concentrations of NAD^+ (open squares). Data represent the means of three measurements. (b) Hanes–Woolf plot of the data in (a).

Fig. 3. Inhibitory effects of NADH (a, b) and NADPH (c, d) on human NADK. The initial velocity (V, U/mg) of the reaction was assayed in the presence of various concentrations of NAD^+ ([NAD^+], mM). (a) Saturation curve for NAD^+ determined in the presence of 0 mM (closed squares), 0.3 mM (open circles), and 0.5 mM (closed circles) NADH. (b) Lineweaver–Burk plot of the data in (a). (c) Saturation curve for NAD^+ determined in the presence of 0 mM (closed squares) and 0.5 mM (open squares) NADPH. (d) Lineweaver–Burk plot of the data in (c).

Online Resource figure legend

Online Resource Fig. S1. Inhibitory effects of NADH (a, b) and NADPH (c, d) on human NADK. (a) Hanes–Woolf plot of the data in Fig. 3a. (b) Eadie plot of the data

in Fig. 3a. The plot was determined in the presence of 0 mM (closed squares), 0.3 mM (open circles), and 0.5 mM (closed circles) NADH. (c) Hanes–Woolf plot of the data in Fig. 3c. (d) Eadie plot of the data in Fig. 3c. The plot was determined in the presence of 0 mM (closed squares) and 0.5 mM (open squares) NADPH.

Table 1

Expression of human NADK.

Host cell	Specific activity (U/mg)[a]	Relative activity (fold)
M15 (pREP4)	0.23	1
Rosetta-gami	1.02	4.4
Rosetta-gami(DE3)	0.79	3.4
Rosetta-gami(DE3)pLysS	4.29	18.7

[a]Specific activity in crude extract.

1

2

Table 2

Improvement in the methods for expression and purification of human NADK.

	Previous study [22]	Present study
Specific activity of expressed human NADK in crude extract	n.r.[a] (0.23 U/mg)[b]	4.29 U/mg
Purification procedure	Three steps (Heat treatment and DEAE as well as nickel–nitrilotriacetic acid column chromatography)	Two steps (Heat treatment and TALON column chromatography)
Yield of purified human NADK	n.r.[a]	15 mg[c]
Specific activity of purified human NADK	n.r.[a]	9.34 U/mg
Homogeneity of purified human NADK	n.r.[a, d]	Homogeneity[d]

[a]n.r.; not reported.

[b]The value obtained in this study (Table 1).

[c]Purified human NADK (15 mg) was obtained from a 13.5 l culture.

[d]N-terminal amino acid sequence of the purified human NADK was not determined in the previous report [22], while homogeneity of this enzyme was confirmed by analysis of the N-terminal amino acid sequence in this study.

3

4

Table 3

Properties of human NADK.

	Previous study [22]	Present study
Subunit molecular mass	49 kDa	43 kDa
Subunit composition (Molecular mass)	Homotetramer (200 kDa)	Homotetramer (172 kDa)
K_m (mM) for NAD^+	0.54	1.07
K_m (mM) for ATP	3.3	-
$S_{0.5}$ (mM) for ATP	-	3.6
n_H	-	1.6
V_{max} (U/mg) for NAD^+	6.7	18.5
V_{max} (U/mg) for ATP	-	18.5
Optimum temperature (°C)	55	50
Optimum pH	7.5	8.0

Table 4

Inhibitory effects of $NADP^+$, NADPH, and NADH on human NADK[a].

Compound	Concentration (mM)	Specific activity (U/mg)[b]	Relative activity (%)
None	0	8.75 ± 0.66	100
$NADP^+$	0.5	8.92 ± 2.26	102
NADPH	0.5	5.83 ± 0.49	66
NADPH	0.3	7.08 ± 0.42	81
NADH[c]	0.5	3.54 ± 0.48	40
NADH[c]	0.3	5.03 ± 0.61	58
NADH[c]	0.1	6.30 ± 0.36	72

[a]NADK activity was assayed in the presence of 1.0 mM NAD^+ and indicated compound.

[b]Means and standard deviations of three independent assays are indicated.

[c]Inhibitory effect of NADH was not attributed to NADH kinase activity of human NADK since the activity assayed in the presence of 0.3 or 0.5 mM NADH was only 10% of NADK activity assayed in the presence of 1.0 mM NAD^+ (data not shown).

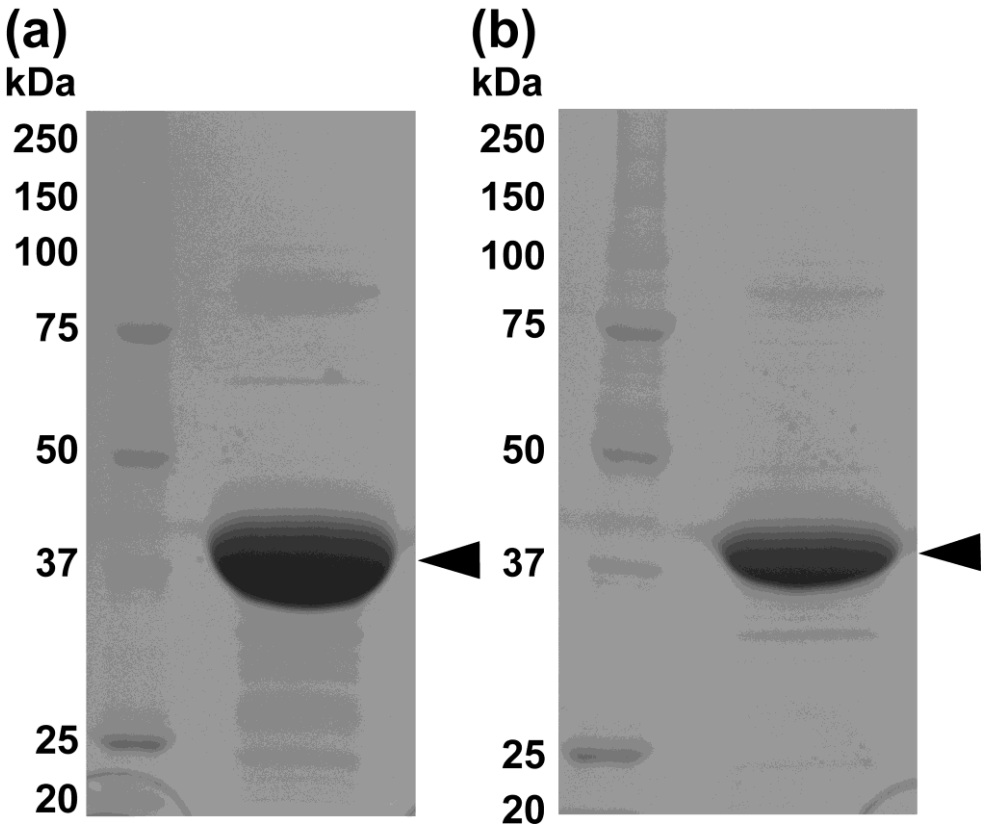
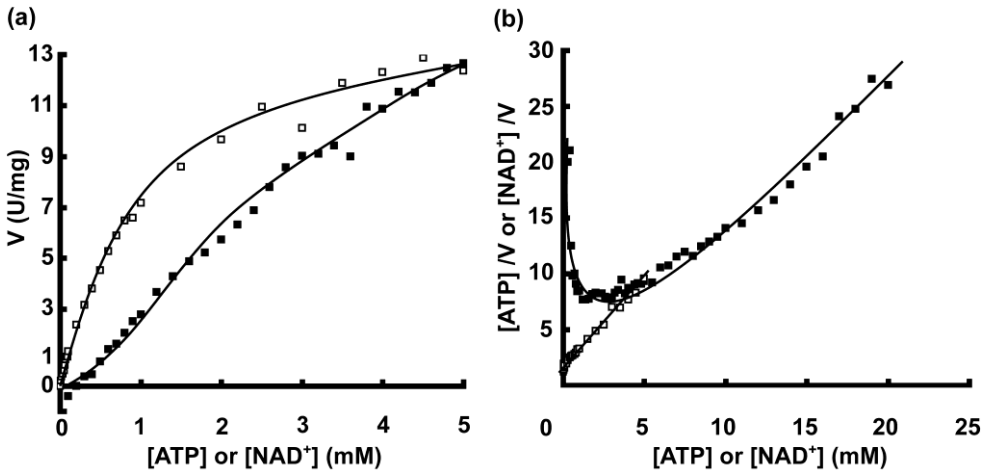


Fig. 1

1



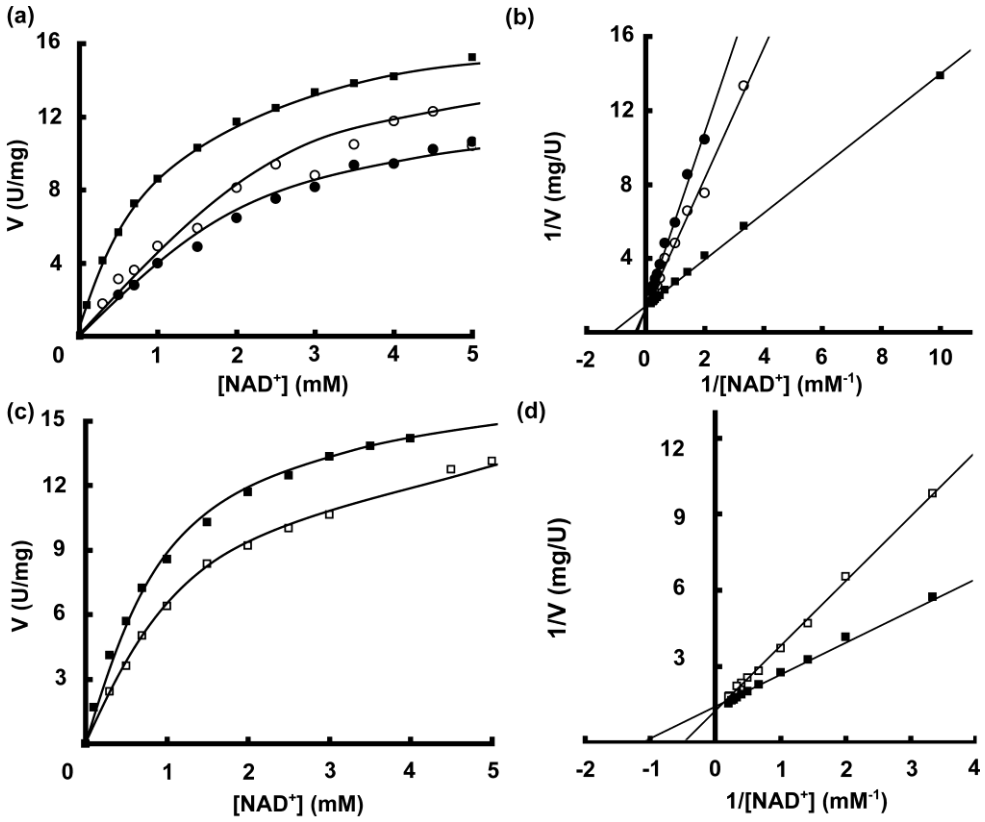
2

3 Fig. 2

4

5

1



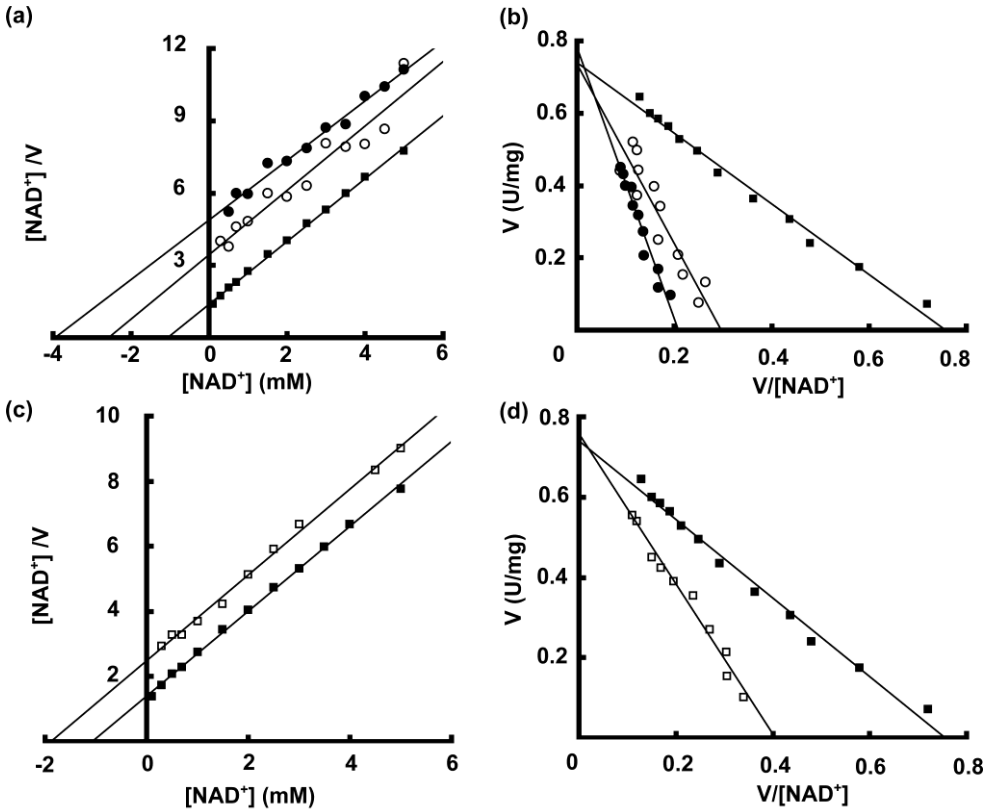
2

3 Fig. 3

4

5

1



2 Supplementary Fig. S1 Ohashi et al.

3